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Original Article

Detection of *Acanthamoeba* and *Toxoplasma* in River Water Samples by Molecular Methods in Iran

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Abstract

Background: Free-living amoebae such as *Acanthamoeba* species may act as carriers of *Cryptosporidium* and *Toxoplasma* oocysts, thus, may play an important role in the water-borne transmission of these parasites. In the present study, a loop mediated isothermal amplification (LAMP) method for detection of *Toxoplasma* and a PCR assay were developed for investigation of *Acanthamoeba* in environmental water samples.

Methods: A total of 34 samples were collected from the surface water in Guilan Province. Water samples were filtrated with membrane filters and followed by DNA extraction. PCR and LAMP methods used for detection of the protozoan parasites *Acanthamoeba* and *Toxoplasma* respectively.

Results: Totally 30 and 2 of 34 samples were positive for *Acanthamoeba* and *Toxoplasma* oocysts respectively. Two samples were positive for both investigated parasites.

Conclusion: The investigated water supplies, are contaminated by *Toxoplasma* and *Acanthamoeba* (oo)cysts. *Acanthamoeba* may play an important role in water-borne transmission of *Toxoplasma* in the study area. For the first time in Iran, protocol of LAMP method was used effectively for the detection of *Toxoplasma* in surface water samples in Iran.

Introduction

Acanthamoeba species may remain viable for many years in environment, even under adverse conditions (1). Some of the *Acanthamoeba* spp. are cases of opportunistic and non-opportunistic infections (2). *Acanthamoeba* spp. are reservoirs and vehicles for the spread of bacteria and this may lead to risks to public health (3). Furthermore, *Acanthamoeba* may act as carriers of *Cryptosporidium* oocysts (4).

Toxoplasmosis is a prevalent zoonotic infection in humans and warm-blooded animals. Mammals and birds serve as intermediate hosts developing tissue cysts in their organs. Infection occurs after ingestion of tissue cysts or contact with soil or water contaminated with oocysts (5). Several water-borne outbreaks of toxoplasmosis have been documented (6-9) and in one instance viable *Toxoplasma gondii* oocysts were found in drinking water (9). *A. castellanii* can engulf *T. gondii* oocysts. Further, internalized oocysts were not digested to a significant extent and retained their ability to establish infection in mice (10).

Several studies for the recovery and detection of *T. gondii* oocysts in contaminated water have been published by different working groups (11-14).

Worldwide loop mediated isothermal amplification (LAMP) had been utilized for a broad spectrum of applications in the biomedical field including the detection of viruses, bacteria, fungi and parasites (15, 16). However, there are few publications about investigating of *Toxoplasma* in water (17-19). The advantage of LAMP method for the detection of *T. gondii* have highlighted and demonstrated in some publications (14, 16, 20-25).

LAMP method was used successfully for detection of *Cryptosporidium* and *Giardia* in water samples in our previous study (26). Determination of these parasites distribution in water resources could assessment the risk of water-borne outbreaks and could be an effective tool for monitoring and preventing of water-borne parasitic outbreaks. Detection of parasites in water is difficult too, so the present study designed to investigate the water-borne parasites, *Acanthamoeba* and *Toxoplasma* in the same aquatic environments.

There is no study about *Toxoplasma* in Iranian water sources, therefore in present study, for the first time LAMP method was used for detection of *Toxoplasma* in the river water samples from Guilan Province in Iran.

Materials and Methods

Study area

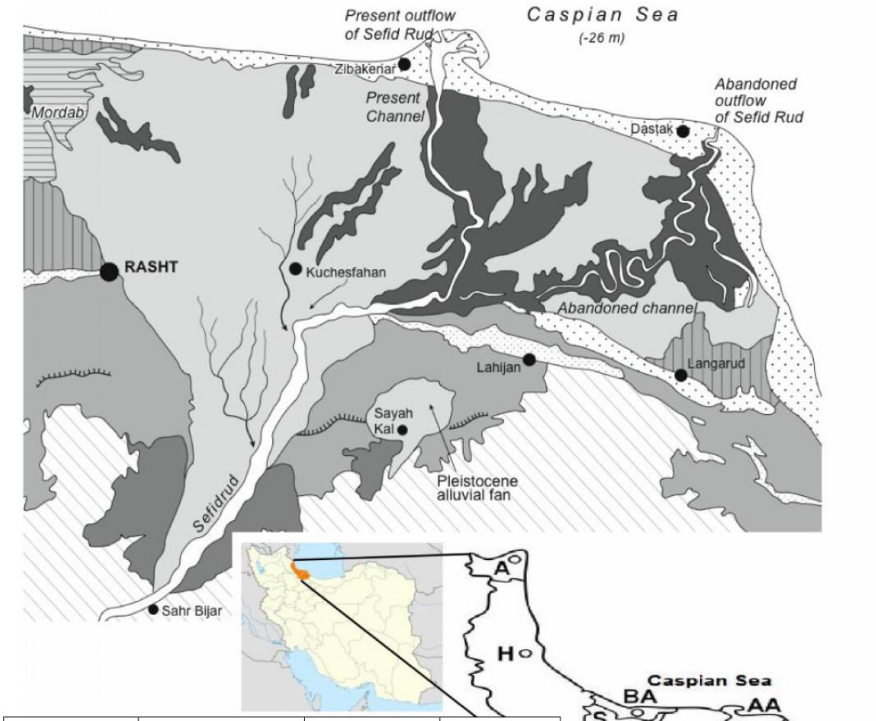
Guilan Province lies along the Caspian Sea. Guilan has a humid temperate climate with plenty of annual rainfall and is known for its moderate, mild, and Mediterranean-like climate. The Sefidrood is a river approximately 670 kilometers long, rising in Guilan and flowing generally northeast to meet the Caspian Sea. The river is Iran's second longest river after the Karun. Zarjoob and Goharrood rivers are two branches of Sefidrood River that across Rasht City and finally meet Bandar-e Anzali Lagoon. Many pollutants as rural, urban and animals' raw sewage threaten water supplies in cities of Guilan (27).

All rivers investigated in present study have different uses including agriculture, industry, residential and recreational activities. The rivers are popular as weekend summer resorts, too. Thirty four water samples were collected during 2009 to 2010 from the river waters in Guilan Province (north of Iran). Some of the sampling sites chosen for sampling in this study were along the Sefidrood River, in different cities of Guilan (Fig. 1), and some samples collected from others surface waters including rivers, dams and lagoons in Guilan (Table 1).

From each sampling point, one to three water samples were collected in 500 ml sterile

bottles for *Acanthamoeba* and 5 liters for *Toxoplasma*. After sampling, the samples transferred to the laboratory of Protozoology, Department of Parasitology and Mycology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran for the study of *Acanthamoeba* by PCR.

The LAMP method was used for the detection of *Toxoplasma DNA* in the Laboratory of Medical and Molecular Parasitology at the Medical school of the University of Cologne, Germany.



Abbreviation	County	Abbreviation	County
BA	Bandar-e Anzali	**R**	Rasht
AA	Astaneh Ashrafiyeh	**Rs**	Roudsar
Lh	Lahijan	**Rb**	Rudbar
Lr	Langerood	**Sh**	seyahkal

Fig. 1: Map of Guilan and study area

Sample collection and preparation

For the isolation of *Acanthamoeba* species, approximately 500 ml of the collected water samples were filtered through cellulose nitrate membrane with pore size 0.45 µm. The filters were transferred on non-nutrient agar plates seeded with Gram-negative bacteria (*Escherichia coli*) as a food source according to our previous study (28). Plates were incubated at room temperature and after 3 days they were microscopically examined for the presence of *Acanthamoeba* cysts or trophozoites. In the absence of amoebae, plates were monitored up to 14 days. *Acanthamoeba* were identified at the genus level, based on morphological characteristics of trophozoites and cysts using light microscopy. For *Toxoplasma*, 5 L environmental water samples were filtrated through

membrane filters (diameter 142 mm) with a pore size of 1.2 µm by means of a vacuum device. Sediment material washed and centrifuged at 1500 g for 15 min. For the removing of particles, the filter was rinsed by 50 ml of 0.1% PBS-Tween 80. This process was repeated two times and particles concentrated by centrifugation at 3000 g for 10 min. The sedimented pellet (1–2 ml, depending on water turbidity) was subjected to sucrose flotation, then the supernatant was subject to DNA extraction (29).

DNA extraction

Acanthamoeba DNA was extracted by phenol–chloroform method according our previously published article(28).

For *Toxoplasma* oocysts, DNA was extracted using the QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's description, with a modification of lyses solution with 15 freeze–thaw cycles in liquid nitrogen and water bath incubation at 65 °C. DNA was eluted in 100 µl elution buffer of the kit used for the LAMP analysis (25, 26).

PCR assay for Acanthamoeba

The *Acanthamoeba* specific primer pairs JDP1 (5'-GGCCCAGATCGTTTACCGTGAA) and JDP2 (5'-TCTCACAAGCTGCTAGGGAGTCA) as described by Schroeder et al. (2001) (30) were used for the amplification of the 500 bp of 18S rDNA gene (31) and Standard PCRs performed as described in our previously paper (28).

The emerging method called LAMP has not only become common currency for the detection of *Toxoplasma gondii* DNA. The method is highly specific, efficient, simple and rapid and the amplification runs under isothermal conditions. No specialized heating equipment is required and the amplification of the target is completed within 60 min (15, 16, 32).

The positive reaction can be visualized either by naked eyes because of the white magnesium pyrophosphate precipitation in the test tube, or by performing the gel electrophoresis (33).

Table 1: Frequency of *Acanthamoeba* cysts and *Toxoplasma* (oo)cysts in surface water samples of Guilan Province, Iran

No	Sampling site	City	Number of Sample at each site	PCR /Lamp results	
				Acanthamoeba	*Toxoplasma*
1	Zarjoob river	Rasht	4	4/4	2/4
2	Eynak Lagoon	Rasht	1	1/1	0/1
3	Goharrood river	Rasht	2	2/2	0/2
4	Sefidrood river	Kiyashahr	4	4/4	0/4
5	Sefidrood river	Astaneh Ashrafiyeh	4	5/7	0/7
6	Sefidrood river	Emamzadeh hashe	2	1/2	0/2
7	Sefidrood river	Manjil	1	1/1	0/1
8	Amlash	Amlash	2	2/2	0/2
9	Langrood	Langrood	1	1/1	0/1
10	Chamkhaleh Lagoon	Langrood	1	1/1	0/1
11	Otaghvar river	Komeleh	2	2/2	0/2
12	Lahijan Lagoon	Lahijan	2	2/2	0/2
13	Siyahkal	Siyahkal	4	4/4	0/4
14	Polrood Dam	Roudsar	1	0/1	0/1
Total			34	30/34	2/34

A primer set used to detect the *Toxoplasma* B1 gene by LAMP method as previously described (14). The reaction was performed as described by Gallas-Lindemann et al. (2013) (25). The results were visualized with UV radiation (PCI-Gel-Imager, Intas, Göttingen, Germany). For each reaction, a positive control [*Toxoplasma* non virulent strain (AHC1), National Research Center Protozoan Diseases, Obihiro University for Agriculture and Veterinary Medicine, Japan] and negative control (double distilled water) were included (25).

Results

Totally 30 (88%) and 2 (5.8%) out of 34 samples were positive for *Acanthamoeba* and *Toxoplasma* respectively. Two samples were positive for all above mention parasites. *Acanthamoeba* species were the most prevalent (Table1). *Toxoplasma* oocysts were detected only in Zarjoob River that flow in Rasht City.

Discussion

Although waterborne parasites, *Acanthamoeba* and *Toxoplasma* have been reported by some researchers, this is the first study to investigate these two parasites in same samples. Because of the predatory activities of different eukaryotic organisms present in aquatic environments, they may serve as potential vectors and paratenic hosts for the environmental transmission of enteric diseases (34, 35). The investigation of protozoan parasites in water is difficult. Therefore establishment of sensitive method for detection of these water-borne parasites are necessary in Iran. An understanding of parasite dynamics in water sources is important for watershed protection. This is the first report on the occurrence of *Toxoplasma* in Iranian water sources. *T. gondii* oocysts have been detected in contaminated water by LAMP method in other countries (25).

Amoebae did not decrease the infectivity and pathogenicity of *T. gondii* oocysts (10). Free-living amoebae are resistant to unfavorable living conditions encountered in nature and to most disinfectants used in the treatment of waters, so they could be resistant (3, 36).

Acanthamoeba spp. was the causative agent of an outbreak associated with a contaminated municipal water supply in the USA and free-living amoeba has been commonly found in various environmental water sources throughout the world (37-41), similar to other studies in Iran (42, 43). *Acanthamoeba* was prevalent in water samples of the study areas and as the results show in the present study *Acanthamoeba* species find more prevalent than another parasite, so could easily uptake *Toxoplasma* oocysts. Therefore may have implications for water quality and public health and may have role in transmission of *Toxoplasma* in the study area.

The present study reveals that the investigated water supplies, which are used for agriculture and camping and some time used by animals, are contaminated by *Toxoplasma* and *Acanthamoeba* species. Zarjoob River was the most pollutant river in study area that was contaminated with both parasites (Table 1). It may because of these surface waters are contaminated more with urban and rural sewages.

Free-living amoebae as *Acanthamoeba* may act as carriers of *Cryptosporidium* and *Toxoplasma* oocysts and, thus, may play an important role in the transmission of cryptosporidiosis and toxoplasmosis (4, 10). In other hand it has also been suggested that the ingestion of oocysts by free-living protozoa and rotifers may have beneficial effects in aquatic ecosystems, such as wastewater treatment systems, as the removal of oocysts would minimize the release of the oocysts into environmental waters from sewage (44).

Conclusion

Free-living amoebae may be regarded as a potential source of *Toxoplasma* oocycts in the study area. They may play an important role in the water-borne transmission of toxoplasmasis in Iran. In addition, additional analysis of isolates is needed to confirm the presence of genotypes, which are infectious to humans.

This is the first report of *Toxoplasma* oocysts in water samples of Iran detected by LAMP method. The method is effective for detection of these water-borne parasites in treated and untreated water samples. It could be used for prevention and hygienic programs. This study can also serve as a platform for further investigations and researches of water sources in Iran.

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